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Multicenter Quality Control of Hepatitis C Virus Protease Inhibitor Resistance Genotyping

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Hepatitis C virus (HCV) protease inhibitor resistance-associated substitutions are selected during triple-therapy breakthrough. This multicenter quality control study evaluated the expertise of 23 French laboratories in HCV protease inhibitor resistance genotyping. A panel of 12 well-defined blinded samples comprising two wild-type HCV strains, nine transcripts from synthetic NS3 mutant samples or from clinical strains, and one HCV RNA-negative sample was provided to the participating laboratories. The results showed that any laboratory with expertise in sequencing techniques should be able to provide reliable HCV protease inhibitor resistance genotyping. Only a 0.7% error rate was reported for the amino acid sites studied. The accuracy of substitution identification ranged from 75% to 100%, depending on the laboratory. Incorrect results were mainly related to the methodology used. The results could be improved by changing the primers and modifying the process in order to avoid cross-contamination. This study underlines the value of quality control programs for viral resistance genotyping, which is required prior to launching observational collaborative multicenter studies on HCV resistance to direct-acting antiviral agents.

Hepatitis C virus (HCV) resistance-associated variants have been shown to emerge rapidly with the use of direct-acting antiviral (DAA) agents with a low-to-moderate barrier to resistance when used as monotherapies (1). Despite significant viral load reductions during administration of the two most advanced protease inhibitors, telaprevir and boceprevir, viral breakthroughs often occur as a result of the outgrowth of variants bearing a number of well-identified amino acid substitutions that confer resistance to these agents (2, 3). The very early selection of resistant viral populations in patients receiving protease inhibitors is in keeping with the preexistence of such variants in most HCV-infected patients before the initiation of treatment. These variants most often preexist in very low proportions and are detectable only by means of next-generation sequencing (NGS) (4–7), whereas they can sometimes be present as major viral populations detectable by means of population sequencing (8–11). In a previous multicenter study, we described the natural genetic variability of the NS3 protease and the presence of variants resistant to protease inhibitors in patients infected with the HCV genotype 1 to 5 strains that are circulating in France in the absence of specific antiviral pressure (12). Although genotyping of HCV resistance to NS3 protease inhibitors has not found a clear indication in clinical practice in the context of the triple combination of pegylated alpha interferon, ribavirin, and either telaprevir or boceprevir, such genotyping is mandatory in clinical trials and cohort studies where these molecules are used, and it might find utility in the coming era of interferon-free regimens. In particular, the impact of such

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substitutions on future retreatment with molecules belonging to the same class is currently not known (13–15). Thus, management of resistance could become an important part of new treatment strategies.

With the goal of gaining confidence in the quality of resistance data collected in multicenter studies, we decided to evaluate the performance of laboratories belonging to the French National Agency for Research on AIDS and Viral Hepatitis (ANRS) Coordinated Action on Hepatitis Virus Resistance to Antiviral Drugs (AC33) in detecting substitutions associated with resistance to protease inhibitors. This article reports the results of this French national quality control program for HCV genotype 1 protease inhibitor resistance genotyping.

MATERIALS AND METHODS

Panel composition. The panel included 12 coded samples: (i) 2 wild-type HCV clinical strains collected from untreated blood donors (one with subtype 1a and one with subtype 1b; viral loads, 6.3 and 7.19 $\log_{10}$ IU/ml, respectively), (ii) 2 clinical strains from patients who failed to respond to boceprevir- and telaprevir-based triple-combination therapy (viral loads, 6.09 $\log_{10}$ and 4.41 $\log_{10}$ IU/ml, respectively), (iii) 7 synthetic mutant NS3 sequences (including two dilutions, neat and 1:100, of the same sequence), and (iv) one HCV RNA-negative sample (Table 2). The centers received the same panel of 12 samples.

NS3 mutants and clinical strain preparation. Synthetic mutants were RNA transcripts constructed from plasmids carrying mutations associated with resistance to HCV protease inhibitors. They were obtained either by direct cloning of the HCV strains from two patients presenting a T54S or a V36M-plus-R155K substitution (quality control no. 10 [QC10] and QC11, respectively; Table 2) or by site-directed mutagenesis conducted on HCV NS3 clones from untreated patients using the GeneArt system (Invitrogen/Life Technologies, Cergy-Pontoise, France) according to the suppliers' recommendations (QC3 to QC9). For each mutant sequence, two complementary oligonucleotide primers spanning the mutation sites were designed. HCV NS3 mutations involved residues 36, 54, 55, 155, 156, and 170. Three subtype 1b mutants were generated with A156S, V170A, and the double T54S-plus-V55A substitution. Three 1a mutants were generated with the single NS3 substitutions V36M and R155K and the T54A-plus-A156T double substitution. All the mutants were amplifiable with the NS3G1FI-M13 and NS3G1RI-M13 primers (12). It is also possible to amplify the samples with the primers Mars F3 and Mars R2 (16), except for the two 1a mutations (V36M [QC3] and T54A plus A156T [QC4]).

Transcription. RNA transcripts were generated for each mutated plasmid according to the technical recommendations relative to the use of the MEGAscript T7 kit and Turbo DNase (Ambion/Life Technologies, Cergy-Pontoise, France). They were diluted in HCV-negative plasma to a final concentration of 10^8 copies/ml. The dilutions were made under RNase-free conditions; 0.5 U/μl of RNasin was added to each mutant sample dilution (recombinant RNasin RNase inhibitor; Promega, Charbonnières, France). Each sample of the panel was aliquoted in 500 μl and subjected to NS3 genotyping according to the recommended protocol (12).

Study design. The panels were anonymized, kept frozen at −80°C, and then shipped under appropriate transportation conditions to the 23 participating laboratories. These laboratories are all central laboratories of university hospitals in major French cities, and they have broad experience in molecular virology. The laboratories were asked to store the samples at −80°C until testing and to use either the NS3 genotype 1 genotyping protocol already described (12) or their own laboratory method. Recommendations for sample handling to avoid contamination were also provided.

The results were to be returned within 6 weeks of receipt of the quality control panel. Upon completion of the testing, the centers were asked to provide the nucleotide and amino acid sequences they generated in text or FASTA format. The laboratories also had to provide details on the genotyping methodology used (extraction, reverse transcription [RT], the use of simple or nested PCR, amplification conditions, and sequencing primers). Finally, the interpretation of the results had to be provided for each sample, including the HCV subtype and the amino acid residue found at NS3 protease positions 36, 41, 43, 54, 55, 155, 156, 158, 168, and 170. The mutation interpretation tool GREG+, developed at the University Hospital of Grenoble and supported within the ANRS network, was distributed to the participants who wished to use it. This software identifies the HCV subtype and interprets protease inhibitor resistance profiles.

Analysis. (i) NS3 sequence alignment. For sequence alignments, the participating centers had to use HCV 1a reference sequence AF009606 (GenBank) and HCV 1b reference sequence BAA18894. Nucleotide and amino acid sequences were aligned for each tested sample, and discrepancies with the synthetic mutant protease sequences were recorded. Differences with the consensus sequence of the tested wild-type strains were also listed (the consensus sequence was recorded as the sequence observed in more than 80% of the sequences received; this cutoff was chosen arbitrarily in order to eliminate errors due to the PCR in a given laboratory).

(ii) Reproducibility. To validate the reproducibility of the NS3 sequence analyses, the panel contained two samples with the same R155K mutant at different dilutions (neat [QC7] and 1:100 dilution [QC8]).

(iii) Sensitivity and specificity. Sensitivity was defined as the proportion of the codons found mutated among truly mutated sites. Specificity was defined as the proportion of the codons found to be wild type among those that were truly wild type. The results were considered falsely negative at a site when a mutation existed at this site and was not found, including as a result of amplification or sequencing defects. The results were considered falsely positive when a negative result was expected; they were considered discrepant when an amino acid different from that of the consensus was found.

(iv) Accuracy. Accuracy was defined as the percentage of correct results among the 12 samples. For codons for which a mixture of 2 to 3 nucleotides was reported, a result was considered correct if the expected mixture was reported or if the expected mutation was present in the mixture.

RESULTS

Participating laboratories and technologies used for sequence analysis. Only 23 laboratories out of 29 that received the samples performed the analyses and returned their results (Table 1). Among them, 15 used automated RNA extraction, 17 performed one-step RT-PCR with superscript III (Invitrogen), 14 performed nested PCR, and 22 used Mars F3 and Mars R2 primers (16) (19 of which had combined the Mars F3 and Mars R2 with NS3GI-M13 primers [12] as recommended in the AC33 protocol). Two centers used their own sets of NS3 protease primers (16, 17).

Performance and reported HCV protease resistance mutations. The majority of the participating laboratories detected most of the NS3 protease inhibitor resistance-associated substitutions. Accuracy, calculated as defined above, ranged from 75% to 100% (Table 2). Twelve centers correctly identified all NS3 protease substitutions. Four laboratories returned one incorrect result each, 5 laboratories returned two incorrect results each, and 2 returned three erroneous results each. For 20 out of 23 laboratories, the nucleotide sequences of the neat and diluted samples containing the same R155K mutant were strictly identical. In one center, the difference was limited to the detection of a mixed population containing the expected substitution; in the two other laboratories, the error was related to contamination of the diluted sample in one case and sequencing troubleshooting in the other case (Table 2). Four centers obtained a wrong subtype result in

TABLE 1 Overview of results

Features of the study	No.
Participants	29
Respondents	24
Answers included in assessment	23[a]
Technical details	
Automated extractions	15
Superscript III users	17
Nested PCR	14
Using AC33 NS3 G1 protocol	19
Using primers NS3 G1	11
Using primers Mars F3 and Mars R2	22
Results 100% right answers	12
Errors	
Mutations	4[b]
Subtype	4[b]
False negatives	12[c]
False positives	2[b]
Contamination	6
Sequencing problems	3[d]

[a] Results from one center were excluded from the analyses.
[b] Contamination with another sample of the panel.
[c] Five V36M, 5 T54A-plus-A156T, and 2 V170A mutants were not sequenced.
[d] Panel samples were amplified, and no NS3 sequence was obtained.

one of the 12 samples of their panel. In all cases, this error resulted from contamination of the sample by another sample with a different subtype.

Four panel samples were amplified, sequenced, and analyzed successfully by all of the laboratories: the 1b A156S mutant, the 1a R155K mutant, and the two 1a clinical strain subtypes with either the T54S or V36M-plus-R155K mutant. Overall, the sensitivity ranged from 66.7% to 100%, and the specificity ranged from 72.2% to 100%.

Incorrect results stemmed from a variety of causes. For the false-negative results, RT-PCR amplification of the HCV NS3 protease failed in 3 samples (7 laboratories): 4 times with the V36M mutant, 5 times with the T54A-plus-A156T double mutant, and twice with the V170A mutant. The V36M and T54A-plus-A156T mutants could not be amplified with the Mars F3 and Mars R2 primers, but they could be amplified with the NS3G1FI-M13 and NS3G1RI-M13 primers. Three of the 4 centers that could not amplify the V36M mutant and 2 of the 5 laboratories that failed to amplify the T54A-plus-A156T double mutant did not use the NS3GI-M13 primers. For the false-positive results, in two centers, the source of the contamination was unknown. For the first one, a positive amplification signal was obtained, but the sequencing failed; for the second one, the contamination probably occurred with the A156S mutant (data not shown). For the discrepant results, 4 centers returned an erroneous substitution in one sample of their panel. In all cases, the nucleotide sequence was from another sample of the panel, suggesting cross-contamination. Nucleotide sequence alignment showed complete identity with another panel sample in 3 cases and a 3-nucleotide difference in one case (data not shown).

Nucleotide and amino acid sequence alignments from the 23 participating centers showed a high sequence identity ratio for each of the six NS3 mutants and with the NS3 consensus sequence from the wild-type and resistant clinical strains included in the panel. No differences in the ability to detect mutated codons were observed according to the type of substitution or between single and double mutants. The centers were also requested to report substitutions at NS3 residues Q41, F43, V158, and D168, known to be associated with decreased HCV susceptibility to protease inhibitors *in vitro* and *in vivo* (18–21). One synthetic mutant carried a V158M substitution, which was detected by all the centers. Globally, when cross-contaminations and amplification issues were excluded, the amino acid sequences reported by the participating centers were identical to the expected ones. At the nucleotide level, mixed populations were reported for both natural strains and synthetic mutants. When mutations other than those present in the samples of the panels distributed were reported, they never involved the protease resistance sites (data not shown).

TABLE 2 Genotyping results from 23 participants in the French national NS3 resistance genotyping quality control study

QC no.[a] or attribute	Sample[b]	Center coding number[c]																						% of correct results
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
QC1	WT/1a	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	SP	X	X	95.6
QC2	WT/1b	X	X	C	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	95.6
QC3	V36 M/1a	X	X	X	SP	/	X	X	X	X	X	X	/	X	X	X	X	X	X	/	X	X	/	X	78.2
QC4	T54A+A156T/1a	X	X	X	/	/	X	X	/	X	X	X	/	X	X	X	X	X	X	C	X	X	/	X	73.9
QC5	A156S/1b	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	100
QC9	T54S+V55A/1b	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	C	X	X	X	95.6
QC6	V170A/1b	X	X	X	X	X	X	X	X	X	X	X	/	X	X	X	X	X	X	X	/	X	X	X	91.3
QC7	R155K/1a	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	100
QC8	R155K 1:100/1a	X	X	X	X	X	X	X	X	X	X	X	X	C	X	X	X	X	X	X	X	SP	X	X	95.6
QC10	T54S/1a[d]	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	100
QC11	V36M+R155K/1a[d]	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	100
QC12	Negative	/	/	/	/	C	/	C	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	91.3
Reproducibility		OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	None	OK	OK	OK	OK	OK	OK	OK	None	OK	OK	
Accuracy (%)		100	100	91.7	83.3	75	100	91.7	91.7	100	100	100	75	91.7	100	100	100	100	100	83.3	83.3	83.3	83.3	100	

[a] QC, quality control.
[b] WT, wild type.
[c] C, contamination; SP, sequencing problem; X, correct mutation(s) and subtype found; /, no amplification; OK, strictly identical results for CQ7 and CQ8; None, discordant results between CQ7 and CQ8.
[d] Clinical strains.

DISCUSSION

No quality control of NS3 protease inhibitor resistance genotyping has been carried out in Europe thus far. This multicenter study demonstrates that laboratories with expertise in sequencing techniques are able to provide reliable HCV protease inhibitor resistance typing. Only 20 incorrect results (0.7%) were reported among the 2,760 amino acid positions analyzed (3 were due to sequencing issues, 11 to false-negative results, 2 to false-positive results, and 4 to discrepant results). The French ANRS AC33 network has developed a tool for HCV NS3 protease inhibitor resistance genotyping and has conducted a multicenter study describing polymorphisms and mutations conferring resistance to protease inhibitors in HCV genotype 1 to 5 strains circulating in France (12). NS3 resistance mutations have proven to be selected when interferon is not sufficiently active in the triple-therapy regimen and the virological response is impaired (5, 22). Their detection before treatment or when virological breakthrough occurs could be important for patient follow-up and still needs to be explored, particularly with the recent wide use of protease inhibitors in clinical settings. Collaborative multicenter studies are needed to evaluate the impact of these resistances and to determine whether this genotyping should be implemented for all patients in clinical practice, as it is for HIV (23). Moreover, setting up a database of DAA resistance mutations will make it possible to compare data from clinical studies with data obtained in the field of clinical practice and will enrich our knowledge of viral resistance and its role in treatment failure in the era of DAA-based therapy.

The national quality control program we established with this goal obtained satisfactory adherence, with 23 complete responses out of 29 centers initially interested in participating. The overall technical quality was good: most of the laboratories were able to amplify the panel samples and to identify the expected protease inhibitor resistance-associated mutations. The best rate of accuracy was achieved in centers using the ANRS AC33 protocol, which combines two sets of NS3 primers. Heterogeneity in the results was mainly related to the methodology used; 12 out of 19 centers using the AC33 protocol succeeded (62.2%). This could be explained by the fact that two samples in the panel could not be amplified when the primers used targeted a region outside of the 645-nucleotide region between 3372 and 4017 (according to HCV H77 1a coordinates). Three out of 23 centers, which used exclusively the Mars F3 and Mars R2 primers that match between NS3 nucleotides 3309 and 4054, were unable to generate results for these two samples. This risk of failure for certain primers was known before the panels were sent to the participating laboratories. One laboratory that did not use the AC33 protocol was able to amplify these two samples because its primers matched the region. Although these primers were highly degenerated, they were close to the NS3G1FI-M13 and NS3G1RI-M13 primers of the AC33 protocol (17).

Another source of false interpretations was the occurrence of cross-contaminations by another member of the panel, as shown in Table 2. The centers had been informed that some panel samples contained high concentrations of transcripts and that recommendations for safe manipulations had been given. Although transcripts and clinical strains might be different in terms of risk of contamination, these errors had the positive effect of encouraging the participating laboratories to review, discuss, and enhance their process to avoid laboratory cross-contaminations.

At the time this national quality control program was started, very few serum samples were available from patients who had been treated with a protease inhibitor and had experienced a viral breakthrough or relapse concomitant with the selection of resistant variants (13–15). In order to obtain a representative panel of possibly selected mutant strains, we decided to construct synthetic mutants carrying most known substitutions associated with protease inhibitor resistance. RNA transcripts were generated and diluted in plasma samples in order to make them relevant to the clinical virology situation. Panel samples covered HCV subtypes 1a and 1b, with a predominance of 1a (6 versus 4) because selection of resistant variants associated with viral breakthrough has been observed more frequently in patients infected with HCV subtype 1a than with subtype 1b (24). Three double mutants were included in the panel, T54A plus A156T, V36M plus R155K, and T54S plus V55A, as well as mutations that confer low-level resistance (3- to 25-fold decrease in susceptibility: V36M, T54A/S, R155K, and A156S) or high-level resistance (>25-fold decrease in susceptibility: A156V/T and V36M plus R155K) to telaprevir and boceprevir *in vitro*. Mutants with V170A and V55A substitutions, preferentially selected by boceprevir (25), were also included in the panel.

In light of our results, it appears that the AC33 protocol is a convenient tool for assessing resistance to HCV NS3 protease inhibitors in clinical studies and clinical practice, either before initiating therapy or at the time of the virological breakthrough or relapse. Protocols are also available for genotypes 2 to 5 (12, 17) and will be evaluated when pangenotypic protease inhibitors become available (26, 27). In addition, previous studies have shown that NS3 protease genotyping is as informative and reliable as NS5B genotyping for determining the HCV genotype and subtype, a finding confirmed in our present study (12, 17).

The participating laboratories were not asked to report an interpretation of susceptibility or resistance to HCV protease inhibitors, because no consensus or resistance algorithm has been defined either nationally or internationally. Moreover, the impact that the results of such testing will have on treatment management is still a matter of debate. However, obtaining resistance information might be important in the future development of therapeutic approaches, including NS3 protease inhibitors. Indeed, some NS3 polymorphisms might impact treatment responses. For instance, patients with a Q80K substitution, frequent in subtype 1a, have been reported to experience virologic breakthroughs, relapses, and lower sustained virological response rates more often when treated with the second-wave protease inhibitor simeprevir (28).

In summary, this first French national multicenter evaluation of HCV protease inhibitor resistance genotyping demonstrated the good quality of the results, with more than 60% of participating laboratories generating 100% accurate results. Improvement of the results resides mainly in using primers designed in a previous study and recommended by the AC33 genotype 1 protocol and following the proper processes to prevent cross-contamination. This work emphasizes the need to develop quality control programs for viral resistance genotyping (29) in order to reduce the heterogeneity of the techniques used in different laboratories and to promote standardized methodological approaches. This

experience was useful to the participating laboratories for assessing their performance and for improving their ability to perform HCV protease inhibitor resistance genotyping, evaluate their own genotyping technique, and implement the AC33 protocol when necessary.

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